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Estradiol enhances liposome-mediated uptake, preferential nuclear accumulation and functional expression of exogenous genes in MDA-MB231 breast tumor cells

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Abstract

Exposure of p53 mutated estrogen-receptor-negative MDA-MB231 human breast tumor cells to a pharmacological concentration of estradiol enhances liposome-mediated uptake and expression of SV-40 luciferase. Unexpectedly, the effect of estradiol on SV-40 expression is evident even when estradiol exposure occurs after the initial uptake phase; this suggests that estradiol may influence gene expression by mechanisms other than increasing gene uptake alone, such as altering the intracellular distribution of the gene. We determined that while uptake of SV-40 luciferase is increased only three-fold by estradiol, there is a 30-fold increase in the nuclear/cytoplasmic ratio of the gene. In order to demonstrate that the influence of estradiol on gene uptake and expression is translated into a functional response, the effects of estradiol on the function of an exogenous gene, in this case the apoptotic function of p53, were assessed in the p53 mutated MDA-MB231 breast tumor cell. While liposome-mediated delivery of CMV-p53 alone was ineffective in promoting cell death, incubation with estradiol and the liposomal p53 complex resulted in a two-fold increase in cell killing over that observed in cells transfected with the corresponding mock vector (empty vector for p53). Evidence that cell killing was occurring through apoptosis included apoptotic body formation, cell shrinkage and an increase in fluorescence after terminal transferase end-labeling. The capacity of estradiol to promote apoptosis in MDA-MB231 cells by a p53–liposome complex is likely to be related to the preferential redistribution of the gene from the cytoplasm to the nucleus which could occur during both the uptake and post-uptake phases. Consequently, although direct effects on gene expression, and the stability of message and protein cannot be ruled out, the predominant effect of estradiol in this experimental system appears to be to influence DNA translocation from the cytoplasm to the cell nucleus. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Estradiol; p53; Breast tumor; Nuclear accumulation; liposome

1. Introduction

Both viral and non-viral systems can be utilized for the delivery of exogenous genes into cells and are being developed for use in gene therapy [1]. The most widely used non-viral gene delivery system relies on the use of cationic lipids to form complexes

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with DNA [2], although non-viral delivery systems are generally relatively inefficient [3]. We have recently reported that the use of pharmacological concentrations of estradiol as well as ionizing radiation can enhance the efficiency of liposomal-mediated gene uptake and expression in the breast tumor cell [4,5]. The current studies were performed to determine how estradiol influences select components of this process including gene uptake, intracellular distribution between the cytoplasmic and nuclear compartments and gene function.

We previously demonstrated [4] that the non-physiological concentration of estradiol used in these studies could increase gene uptake and expression in both estrogen-receptor-positive MCF-7 and estrogen-receptor-negative MDA-MB231 breast tumor cells [6]. This finding is consistent with an earlier report indicating that the effects of pharmacological concentrations of estradiol are not dependent on the estrogen-receptor status of the breast tumor cell [7]. The current work is focused exclusively on MDA-MB231 cells as these cells have mutant p53 [8] and mutations in p53 have been shown to prevent apoptosis [9,10]. Since adenoviral delivery of p53 has been shown to promote apoptosis in p53 mutant MDA-MB-231 cells [11], we reasoned that these cells could be used to assess gene function if the delivery of p53 in the presence of estradiol were to promote apoptosis.

The present studies demonstrate that estradiol increases gene uptake, and produces a profound alteration in the intracellular distribution of an exogenous gene. Furthermore, the function of the exogenous gene remains intact as these effects of estradiol are accompanied by the induction of apoptosis when the exogenous gene delivered to the cell is p53.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM, 56–439) was obtained from Hazelton Research Products, Denver, PA; L-glutamine, penicillin (10 000 U/ml), streptomycin (10 mg/ml), and fetal bovine serum were obtained from Whittaker Bioproducts, Walkersville, MD; defined bovine calf serum was obtained from Hyclone Laboratories, Logan, Utah. Trypsin-EDTA, lipofectamine, and optiMEM were obtained from Gibco-BRL (Gaithersburg, MD). The pSV-40–luciferase (Promega) was kindly provided by Dr. Phil Hylemon. The p-CMV-p53 and p-CMV-mock p53 were constructed as previously described [9].

2.2. Cell culture

The MDA-MB-231 cells were obtained from Dr. Eric Westin at the Medical College of Virginia of Virginia Commonwealth University, Richmond, VA. Cells were maintained in Dulbecco's minimal essential medium (Hazelton Research Products, Denver, PA) supplemented with 5% fetal calf serum (Life Technologies, Grand Island, NY), 5% defined bovine serum (Hyclone Laboratories, Logan, UT) glutamine (29.2 mg/l00 ml), amphotericin B (5 μg/ml) (Sigma), and penicillin/streptomycin (0.5 ml/100 ml) (Whittaker Bioproducts, Walkersville, MD). Approximately, 1×10^4 MDA-MB-231 cells per well were subcultured in six-well plates and allowed to grow for 2–3 days so as to achieve 60% confluency prior to conducting the gene delivery experiments described below.

2.3. Preparation of DNA–liposome complex and optimization of transfection conditions

The manufacturer's recommendations were followed for DNA–liposome complex preparation and optimization of the transfection conditions to human breast cancer cells. Briefly, DNA was mixed with lipofectamine in serum free optiMEM media and incubated at room temperature for 45 min with gentle shaking every 15 min. Optimal conditions for transfection proved to be the use of 6 μl of lipofectamine. As 2–4 μg of DNA per ml gave adequate results, 2 μg of DNA (1:1::SV-40 luciferase: CMV-β-galactosidase) and 6 μl of lipofectamine [4,5,12] were used throughout the study.

2.4. Transfection procedure and estradiol co-treatment

Estradiol was dissolved in a mixture of ethanol/polyethylene glycol (at a ratio of 45:55), a vehicle non-toxic to human breast cancer cells [13]. MDA-

MB-231 cells in six well plates were washed with optiMEM, exposed to the DNA–liposome complex in the presence of either vehicle or various concentrations of estradiol (in a volume of 1 ml) and incubated at 37°C for 5 h. An additional 1 ml of MEM media containing 20% serum and either vehicle or estradiol was added and the incubation continued overnight at 37°C. The media was decanted and replaced with MEM containing 10% serum and cells were incubated at 37°C for the indicated times. In selected experiments, the cells were exposed to estradiol either during the uptake or post-uptake phases, as well as during both uptake and post-uptake phases, as shown in Fig. 1.

2.5. Luciferase reporter assay

Cells transfected with DNA were washed twice with 2 ml PBS, and lysed using 250 μl/well of reporter lysis buffer (Promega, Madison, WI) containing 125 mM Tris, pH 7.8 with H_3PO_4, 10 mM EDTA, 10 mM DTT, 50% glycerol and 5% triton X-100, diluted 1:4 for 15 min at room temperature. The cell lysate was scraped using a rubber policeman, collected in 1.5 ml microfuge tubes and centrifuged at 10 000 rpm for 2 min at 4°C. The supernatant was transferred to a 1.5-ml Eppendorf tube and stored at −70°C prior to the determination of luciferase activity.

The luciferase activity of the cellular extract was determined by mixing 20 μl of cell extract with 100 μl of Promega Luciferase reagent containing 270 μM coenzyme A (lithium salt), 470 μM luciferin, 530 μM ATP, 20 mM tricine, 1.07 mM $(MgCO_3)$ $4Mg(OH)_2{\cdot}5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA and 33.3 mM DTT, pH 7.8 at room temperature. Relative light units (RLU) were measured for 20 s in a Berthold LB 9501 luminometer [4,5,10].

In parallel experiments, the transfected cells were trypsinized to quantitatively evaluate viable cell number by trypan blue exclusion [14]. The luciferase activity was expressed as RLU per viable cell.

2.6. Cell fractionation

For the determination of intracellular distribution of the transfected gene, the transfected cells were pelleted and processed as described below. The cells were labeled with [^{3}H]thymidine at 37°C for 24 h. The radioactive media was decanted and the cells were pelleted and suspended in 1 ml triton based buffer (10 mM Tris-HCl, 10 mM KCl, 1.5 mM $MgCl_2$, 0.25% triton). Cells were homogenized by repeated passage (×5) through a 26.5-gauge needle attached to a 1-ml disposable syringe. The solution was centrifuged at 10 000 rpm for 10 min. The supernatant (cytoplasmic fraction) was removed and the nuclear pellet was resuspended in sucrose based buffer (0.25 M sucrose, 10 mM NaCl, 10 mM Tris HCl, 1.5 mM $MgCl_2$) together with 150 μl of detergent (10% sodium deoxycholate and 10% Tween-40) and vortexed for 30 s to lyse nuclei. The purity of nuclear and cytoplasmic fractions were verified, respectively, based on radioactive thymidine incorporation by tricholoroacetic acid precipitation and colorimetric assessment of lactate dehydrogense enzyme activity.

2.7. Extraction and quantitation of transfected DNA

The luciferase plasmid DNA was extracted from MDA-MB231 breast cancer cells utilizing the standard Hirt protocol [15] which extracts low molecular weight DNA through the preferential precipitation of cellular DNA in the presence of SDS and NaCl. Briefly, the transfected cells were suspended in 0.045 M Tris-borate, 0.001 M EDTA, 0.5% SDS and 1.6 M sodium chloride and digested overnight at 4°C. The cellular extract was pelleted and DNA was extracted twice with 200 μl of PCI (phenol/chloroform/isoamyl alcohol, 25:24:1) and once with chloroform. The DNA was purified and precipitated using two volume of ethanol, 0.02 M sodium chloride, incubated at −80°C for 30 min and then pelleted at 14 0000 rpm for 15 min at 4°C. The DNA was resuspended in an appropriate volume of sterile water in proportion to the viable cell number so as to maintain equivalent DNA concentrations in control and irradiated cells. DNA extracted from approximately 500 000 cells was loaded into each lane of an 0.8% agarose gel in TBE buffer containing ethidium bromide, and electrophoresis was performed at 90 mV for 3–4 h. Densitometric analysis was performed to quantitate the extracted DNA in each lane.

2.8. Statistical analysis

All experiments were repeated at least once. The estradiol-treated cells were compared with controls by ANOVA; a P-value of <0.05 was considered to be statistically significant. The statistical analysis was performed utilizing Statview 512TM McIntosh statistical software.

3. Results

3.1. Influence of estradiol exposure during liposome-mediated gene uptake and post-uptake phases on gene expression

We have previously reported that exposure of either estrogen-receptor positive MCF-7 or estrogen-receptor negative MDA-MB231 breast tumor cells to a pharmacological concentration of estradiol increases the expression of luciferase from an SV-40–luciferase reporter gene transfected into the cells by a liposome-mediated transfection protocol [4]. These studies were performed as shown by arrow C in Fig. 1, with continuous exposure of the cell to estradiol during both the uptake phase (when cells are exposed to the liposomal DNA complex) as well as during the post-uptake phase (after the liposomal DNA complex has been withdrawn from the incubation medium).

Estradiol may influence subcellular processes in cytoplasmic and nuclear compartments[7,16] as well as cellular membrane stability [17]. Thus, it is possible that the observed effects of estradiol represent alterations in the intracellular distribution of the exogenous gene as well as enhanced gene uptake across the cell membrane. If this is the case, then estradiol should be capable of influencing gene expression even during the post-uptake phase, when effects on uptake across the membrane are likely to be negligible.

In order to determine whether the influence of estradiol on gene expression could be related to translocation and expression independent of gene uptake, we evaluated the effects of estradiol on gene expression with estradiol exposure during the post-uptake phase (i.e. after removal of the liposomal complex as shown by arrow B in Fig. 1). We further compared

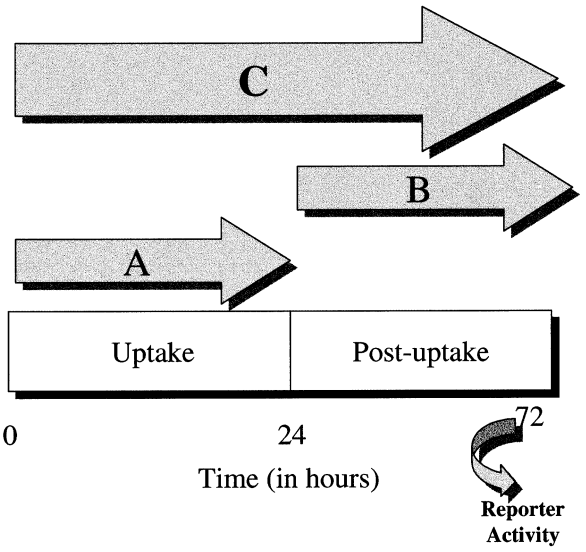

Fig. 1. Schematic representation of the protocol for liposomal gene delivery combined with estradiol in MDA-MB-231 human breast cancer cells. Phases of gene-delivery: transfection with the liposomal SV-40–luciferase gene was initiated at time 0 and continued at 37°C for 24 h (uptake phase). After the gene formulation was removed and replaced by fresh medium, incubation was continued for an additional 48 h (post-uptake phase). After 72 h the cell extract was prepared to evaluate reporter activity. Cells were exposed to estradiol during the uptake phase alone (A), the post-uptake phase alone (B) or during both the uptake and post-uptake phases (C).

the relative effects on gene expression of estradiol exposure during both the uptake and post-uptake phases (arrow C in Fig. 1) with estradiol exposure during either phase (arrows A and B) alone.

Fig. 2 shows that, as expected, luciferase expression from the SV-40–luciferase reporter gene was increased by exposure to estradiol during the uptake phase. In addition, a significant increase in luciferase expression was also observed upon exposure to estradiol during the post-uptake phase. As would be expected from these and previous observations [4], the increase in expression following exposure to estradiol during both the uptake and post-uptake phases was greater than that with estradiol treatment during the uptake or post-uptake phases alone.

3.2. Influence of estradiol on intracellular gene distribution

The observation that estradiol exposure even during the post-uptake phase increases gene expression

supports the hypothesis that estradiol is having effects other than or in addition to enhancing gene delivery across the cell membrane. In order to determine how estradiol influences levels of the exogenous gene within the cell as well as its intracellular distribution, cells were transfected with the SV-40–luciferase–liposomal complex either with or without a 24-h co-exposure to estradiol. SV-40 luciferase was isolated from whole cell extracts as well as the nuclear and cytoplasmic fractions. The relative purity of the cytoplasmic and nuclear fractions was substantiated by demonstrating that greater than 95% of cellular lactate dehydrogenase activity was associated with the cytoplasmic extract and similarly that greater than 95% of [^{3}H]thymidine incorporation was evident in the nuclear extract (Fig. 3).

In the representative experiment assessing total gene uptake into the cell presented in Fig. 4A, cells treated with estradiol during the uptake phase are shown to accumulate significantly higher levels of the SV-40–luciferase transgene than the corresponding controls transfected with SV-40 luciferase in the absence of estradiol. Fig. 4B indicates that there was a 2.8 ± 0.9-fold increase in the delivery of the luciferase gene by estradiol.

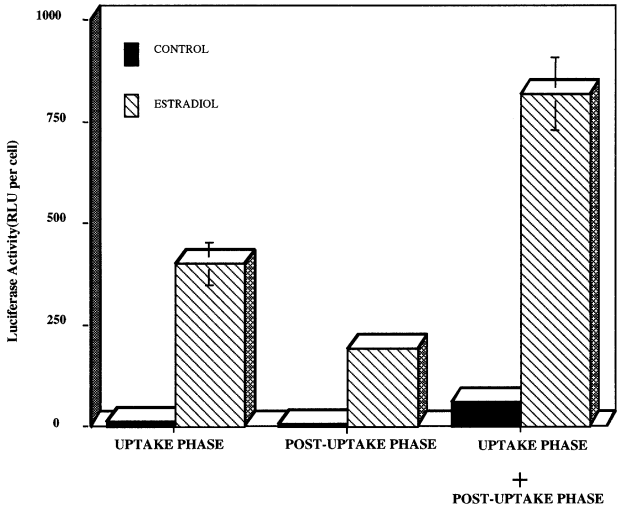

Fig. 2. Improvement of SV-40–luciferase gene expression in MDA-MB-231 human breast cancer cells following exposure to 100 μM estradiol during the uptake, post-uptake, and uptake/post-uptake phases. Note that maximal improvement in luciferase expression occurs following exposure to estradiol during both the uptake and post-uptake phases.

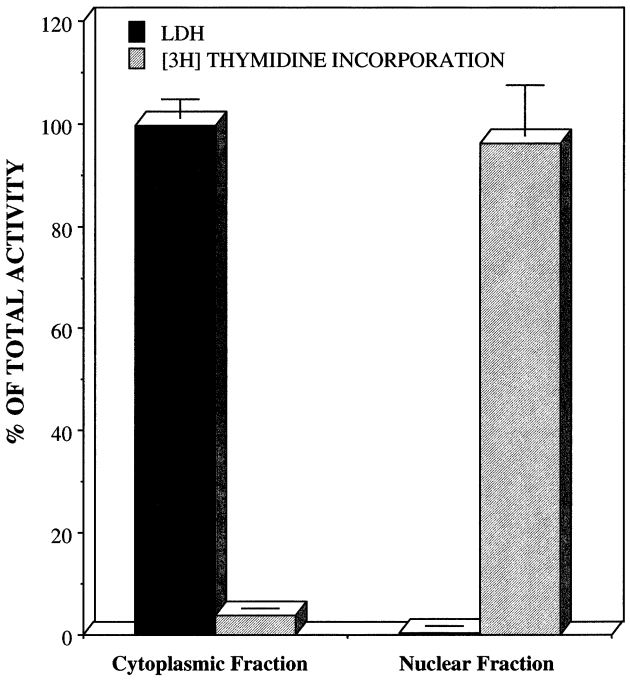

Fig. 3. Cytoplasmic and nuclear fractionation of cells. Lactate dehydrogenase and [^{3}H]thymidine incorporation were used as cytoplasmic and nuclear markers for assessing the purity of the separated fractions from which the exogenously transfected DNA was extracted (as shown in Fig. 5).

Although there is a significant increase in gene uptake, this is clearly insufficient to explain 30-fold increase in gene expression which occurs as a result of estradiol exposure during the uptake phase. In the studies presented in Fig. 5A to establish whether estradiol affects the nuclear to cytoplasmic distribution of the exogenous transgene, it is evident that estradiol essentially reversed this distribution ratio. That is, a far greater amount of the luciferase gene accumulated into the nucleus of estradiol-treated cells as compared to those treated with the vehicle control. Correspondingly, a marked decrease was evident in cytoplasmic SV-40–luciferase in the estradiol-treated cells. The alteration in intracellular distribution of the luciferase gene from the cytoplasm into the nucleus was quite pronounced. Fig. 5B indicates that there was a 28.8 ± 0.9 fold increase in the nuclear/cytoplasmic ratio of the SV-40–luciferase which approximates the increase in gene expression shown in Fig. 2.

3.3. *Promotion of apoptotic cell death in MDA-MB231 breast tumor cells by the combination of estradiol and the liposomal p53 complex*

The studies described above demonstrate increased gene uptake, redistribution of the gene from the cytoplasm to the nucleus and enhanced gene expression by estradiol. However, it is necessary to demonstrate that these effects of estradiol also serve to re-establish the functional response of the gene and that this function is pharmacologically significant. For this purpose, we chose to assess the capacity of estradiol to re-establish the apoptotic function of p53, as MDA-MB231 cells have a mutant p53 gene [8]. We reasoned that restoration of p53 to these cells might be sufficient to promote apoptotic cell death as has

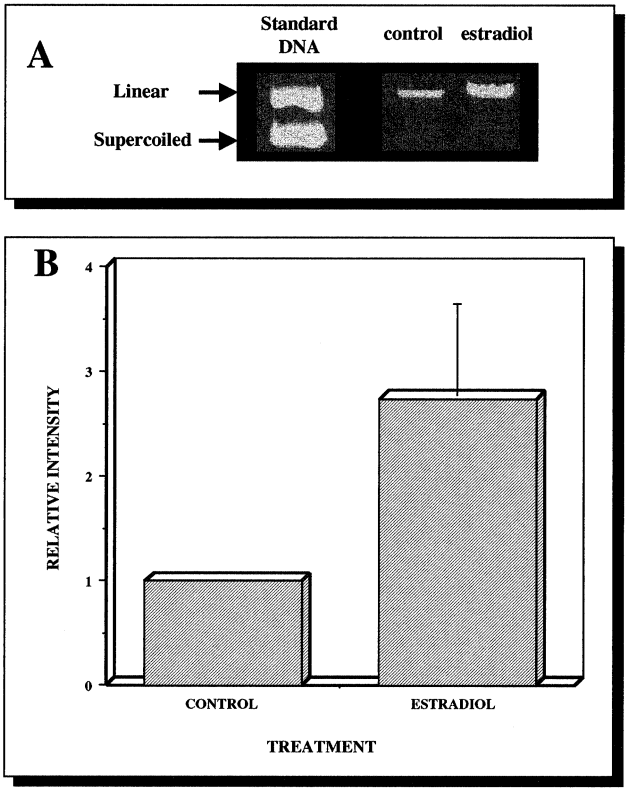

Fig. 4. Estradiol improves delivery of the SV-40–luciferase gene in MDA-MB-231 human breast cancer cells. Cells were exposed to 100 μM estradiol and the liposomal SV-40–luciferase complex for 24 h. (A) SV-40–luciferase plasmids isolated from the entire cells and separated by agarose gel electrophoresis. (B) The extent of improvement in luciferase gene delivery from three different experiments ± S.E.M.

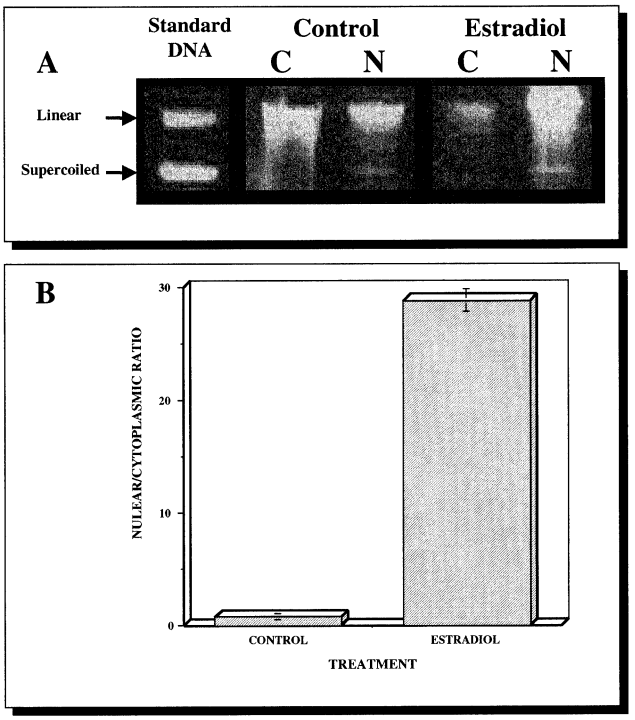

Fig. 5. Estradiol alters the intracellular distribution of the SV-40–luciferase gene in MDA-MB-231 human breast cancer cells. Cells were exposed to 100 μM estradiol and the liposomal SV-40–luciferase complex for 24 h. (A) SV-40–luciferase plasmids extracted from cytoplasmic and nuclear fractions. (B) The nuclear/cytoplasmic ratio for the luciferase gene in two separate experiments ± S.E.M.

been shown in other studies where wild-type p53 was restored to p53 mutant cells [11]. Cells were transfected with either p53 under the control of a constitutive CMV promoter or with mock p53 (the plasmid

Table 1
Re-establishing p53-mediated cell death in MDA-MB-231 cells. MDA-MB-231 cells were treated with the liposomal p53 complex and then continuously exposed to estradiol (100 μM) during uptake and post-uptake phases:

Treatment		
Chemical	Gene	Dead Cells (%)[a]
None	p53	31.5 ± 0.2
Vehicle	Mock p53	36.6 ± 9.4
Vehicle	p53	31.5 ± 0.2
Estradiol	Mock p53	40.5 ± 0.2
Estradiol	p53	80.2 ± 3.8[b]

[a]Values represent the means of duplicate samples.
[b]This value was significantly higher than all other samples ($p < 0.05$).

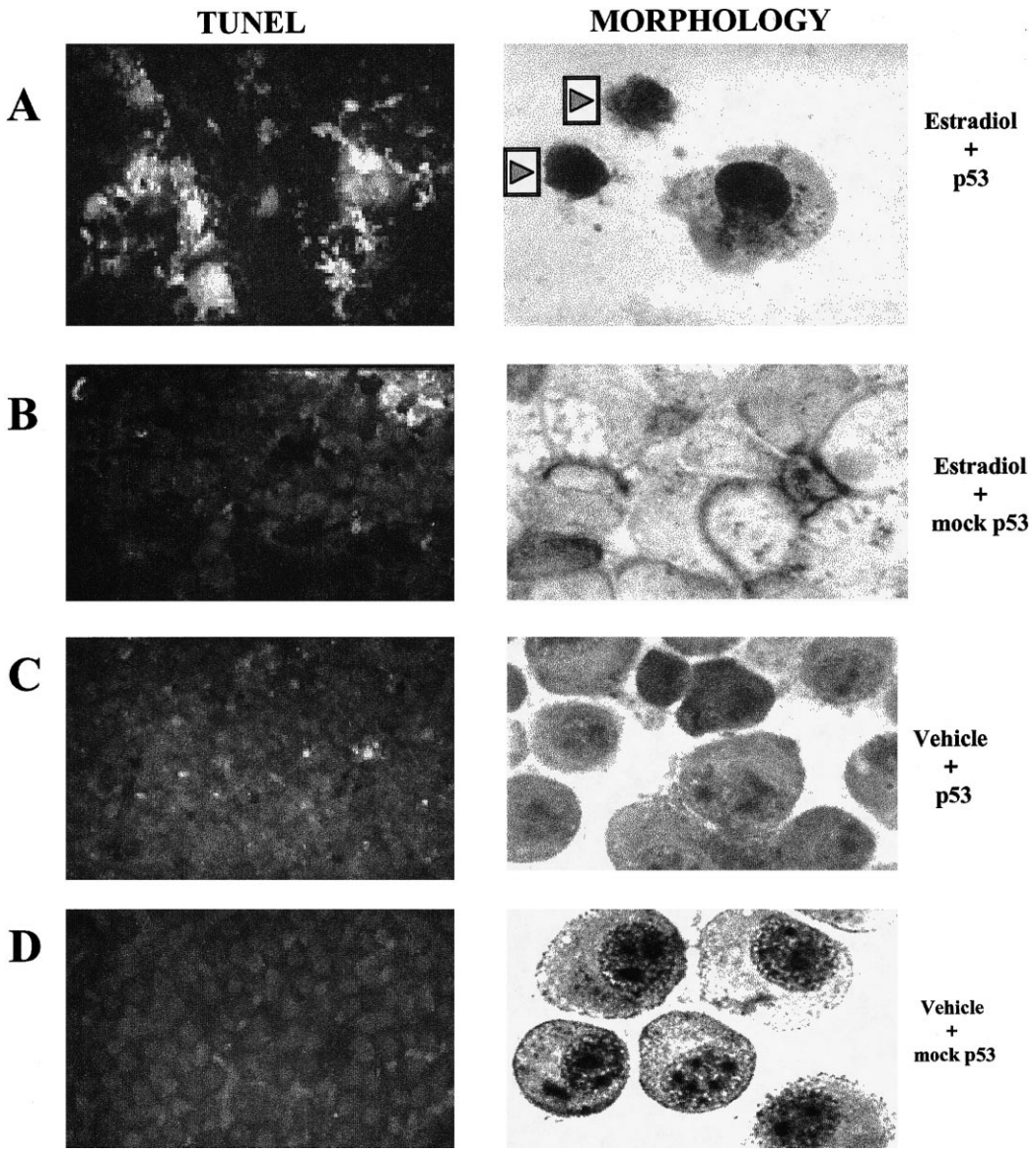

Fig. 6. Fluorescent staining (TUNEL assay) and morphological evaluation (phase contrast microscopy) of apoptotic cell death in MDA-MB-231 human breast cancer. Cells were treated with the liposomal CMV-p53 gene complex and estradiol (100 μM) during both the uptake and post-uptake phases. For the TUNEL assay, cells were incubated with fluorescent labeled antibodies. For morphological evaluation, cells were incubated with Geimsa–Wright stain. Note formation of apoptotic bodies (arrowhead) in the cells treated with both estradiol and the p53 gene (A). These typical characteristics of apoptosis were negligible in the cells treated with estradiol/mock p53 (B), vehicle/p53 (C) or vehicle/mock p53 (D).

originally used for cloning the p53 construct) using the standard Lipofectamine transfection protocol, but without estradiol treatment. Table 1 indicates that there was no difference in the extent of cell death (30–35% of the cell population) in the absence or presence of p53 under these experimental conditions (i.e. transfection in the absence of estradiol). We concluded that the observed toxicity was related to the transfection protocol and that p53 was not influencing cell viability. The lack of a functional p53 response was likely to be due to insufficient levels of the gene within the cell or limited distribution of the gene to the nucleus.

Consequently, cells transfected with the liposome–p53 were incubated with estradiol during both the uptake and post-uptake phases in order to both in-

crease levels of the gene within the cell and to enhance its translocation to the nucleus. Under these conditions, cells transfected with p53 demonstrated a marked enhancement of cell killing. Table 1 indicates that incubation with estradiol essentially doubled the number of non-viable cells as compared to transfection with p53 in the absence of estradiol. Although exposure to estradiol during either the uptake or post-uptake phases alone significantly enhances reporter transgene expression (as shown in Fig. 2), neither of these approaches was sufficient for the induction of apoptosis (data not shown).

To ascertain that cell death which occurred after transfection with p53 in the presence of estradiol was indeed related to an apoptotic mechanism, we evaluated various morphological and biochemical indicators of apoptosis. As shown in Fig. 6, phase contrast microscopy clearly indicated the presence of shrunken cells and apoptotic nuclear bodies after treatment with estradiol and transfection with p53. These characteristics were negligible in the MDA-MB-231 cells treated with estradiol and a mock p53 vector (panel B), the vehicle and p53 (panel C), or with vehicle and mock p53 vectors (panel D). Similarly, the TUNEL assay demonstrated an intense fluorescent signal in the MDA-MB-231 cells treated with estradiol and p53 (panel A), which was not evident for any of the other experimental condition.

4. Discussion

Previous studies in this laboratory [4] have demonstrated that pharmacological concentrations of estradiol increase the expression of genes delivered by a liposome-mediated protocol in both estrogen-receptor-positive MCF-7 and estrogen-receptor-negative MDA-MB231 breast tumor cells [6]. These observations indicate that the effects of a supra-physiological concentration of estradiol on gene uptake and expression in the breast tumor cell is independent of estrogen-receptor status. In the current work, we have further established that the mechanism for improving transgene expression by estradiol includes its influence on gene uptake across the cell membrane as well as redistribution of the transgene from the cytoplasm into the nucleus. In fact, the most pronounced effect of estradiol appears to be the 30-fold increase in the nuclear to cytoplasmic ratio rather than the 2–3-fold increase in gene uptake. The capacity of estradiol to alter the nuclear to cytoplasmic distribution of the gene is further supported (albeit indirectly) by the observation that estradiol enhances gene expression during the post-uptake phase – when transport of the gene across the cell membrane is expected to be negligible.

A number of mechanisms could be responsible for the effects of estradiol on transmembrane gene transport, the redistribution of the transgene between the nuclear and cytoplasmic compartments and the enhancement of gene expression. These include effects on cell membrane stability [17], deregulation of ions such as calcium [16], alteration in the nuclear membrane [16] and of cyto/nuclear-skeletal proteins [7,18]. Estradiol could also influence the function of the exogenous gene at the transcriptional level by stimulating activity of the promoter, post-transcriptionally at the level of mRNA stability or at the translational and post-translational levels, respectively, by increasing protein synthesis or the stability of the expressed protein.

It is of interest that apoptotic cell death could not be re-established simply through liposomal-mediated delivery of the p53 transgene to the MDA-MB231 cells. This could be related to the fact that most of the exogenous DNA is localized to the cytoplasmic fraction. However, when cells were exposed to estradiol during either the uptake or post-uptake phases alone, the liposomal p53 complex failed to promote apoptosis. This suggests that despite the effects of estradiol on the redistribution of the gene from the cytoplasm to the nucleus, there may still have been insufficient p53 in the cell to promote apoptosis. In contrast, when MDA-MB231 cells were exposed to 100 μM estradiol both immediately upon transfection with p53 as well as during the post-uptake phase, the cells apparently underwent apoptotic cell death. Thus, it appears that a threshold level of nuclear p53 gene expression may be required for the promotion of apoptotic cell death.

In summary, these studies indicate that exposure of the breast tumor cell to a pharmacological concentration of estradiol increases the liposome-mediated uptake, cytoplasmic to nuclear translocation, expression and function of an exogenous gene in the breast tumor cell. Therefore, estradiol has the

capacity to alter fundamental aspects of cell function through estrogen-receptor-independent mechanisms.

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